

Improvements in Magnetic Polymer Particles

This invention relates to magnetic polymer particles carrying a chelating matrix loaded with a metal as well as to a process for the preparation of magnetic polymer particles carrying said chelating matrix. In particular, the invention relates to magnetic polymer particles carrying a carboxymethylated aspartate (Cm-Asp) chelating group and to the coupling of the Cm-Asp group with the magnetic polymer particle.

Magnetic polymer particles are of general utility in various medical and biochemical fields, for example as transport vehicles for the delivery of pharmaceutical products, for diagnostic purposes, for separation and for synthetic purposes. Such particles rely upon their magnetic properties in order to perform these functions: in diagnostic assay applications, for example, application of a magnetic field to a sample containing an analyte bound to magnetic polymer particles allows the isolation of the analyte without the use of centrifugation or filtration; and in therapeutic applications, for example, application of a magnetic field to the patient may serve to target drug-carrying magnetic polymer particles to a desired body site.

By magnetic is meant herein that the polymer particles contain superparamagnetic crystals. Thus the magnetic polymer particles are magnetically displaceable but are not permanently magnetizable. Many processes for preparing magnetic polymer particles are known, a large number of which involve preparing maghemite- or magnetite-containing polymer particles from pre-formed magnetic iron oxides, e.g. magnetite. Some of processes involved are described in US-A-4,654,267 (Ugelstad) the contents of which are incorporated herein by reference.

The use of immobilised metal ion affinity chromatography (IMAC) has been known for many years. The IMAC purification process is based upon the

employment of a chelating matrix loaded with transition metal ions such as Cu^{2+} or Ni^{2+} which is capable of binding electron donating groups present on the surface of proteins, in particular the imidazole side chain of histidine. The electron donating group is believed to coordinate to vacant coordination sites around the metal ion. The interaction between the metal ion and the electron donating groups present on the protein surfaces can be altered by, for example, varying pH and hence purification can be achieved via reversible metal complex/protein interaction. Most commonly, if a protein is bound to a solid phase via the interaction between the metal ion and the imidazolyl side chain of histidine, the protein can be removed by addition of imidazole itself, i.e. by competitive elution.

Several different chelating ligands have been employed in IMAC to purify proteins. Nitrilo triacetate (NTA) (a tetradentate ligand) and the pentadentate ligand tris(carboxymethyl)ethylenediamine are examples of such ligands but these suffer from various disadvantages such as unspecific protein interaction, metal leakage etc.

US 6242581 proposes a solution to the metal leakage problem by the use of a carboxymethylated aspartate (Cm-Asp) group in IMAC where the bound transition metal ion has octahedral geometry. The ligand is said to be ideal for isolating histidine tagged recombinant proteins. Other advantages of Cm-Asp are discussed in US 5962641, e.g. resistance to reducing agents.

In these Patents the Cm-Asp ligand is bound to an agarose solid phase which is preferably cross-linked although other polymer matrices such as polystyrene, nylon and SEPHAROSE are suggested. Whilst these matrices may be magnetic the magnetic particles do not remain in suspension and the solid phases are therefore of limited use in assays.

It has now been surprisingly found that the Cm-Asp

chelating ligand can be coupled to a magnetic polymer particle giving rise to a moiety that possesses the ability to bind histidine-tags in recombinant proteins or His, Cys, Met, Gln, Asn, Lys or Tyr residues present in metalloprotein active sites in native proteins or peptides, the ability to bind phosphorylated proteins or peptides and also magnetism. This allows the skilled biochemist more flexibility in his assaying procedures.

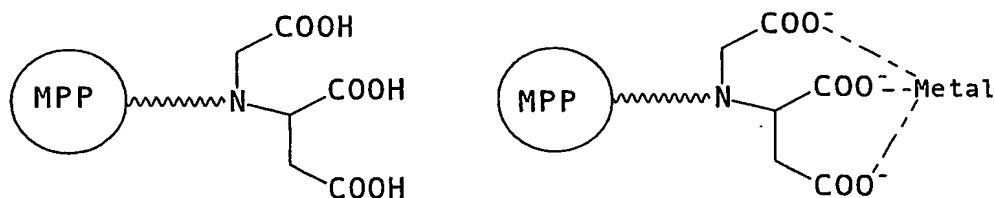
The inventors have also devised ways to couple the Cm-Asp ligand to the magnetic polymer particles in high yield thereby producing an excellent IMAC agent.

Viewed from a first aspect, therefore, the present invention provides a conjugate comprising a magnetic polymer particle bound to a carboxymethylated aspartate chelating ligand.

Viewed from another aspect the invention provides a conjugate comprising magnetic polymer particle bound to a carboxymethylated aspartate ligand chelating a metal atom or ion.

Viewed from another aspect the invention relates to a process for the preparation of a conjugate as hereinbefore defined comprising reacting a magnetic polymer particle with a Cm-Asp chelating ligand.

The Cm-Asp ligand bound to the magnetic polymer particle (MPP) is depicted below both in its uncoordinated state and coordinated to a metal (the wavy line representing a bond or a linker between the Cm-Asp and particle):



The magnetic polymer particles used in the process of the invention may be any magnetic polymer particle e.g. as described in US-A-4,654,267. The particles are preferably porous to allow the presence of the
5 superparamagnetic crystals in the pores thereof. The surface of the magnetic particles is normally functionalised to allow coupling of the Cm-Asp ligand to the polymer particle, e.g. it may be functionalised to carry any known surface structure such as carboxyl
10 groups, tosyl groups, amino groups, epoxy groups, maleamido groups, thiol groups etc. Hence, the surface may be amine functionalized before Cm-Asp coupling. Alternatively, an amine functionalised surface can itself be further functionalised to carry other
15 functional groups, e.g. COOH groups.

The polymer particle is preferably made from combinations of vinylic polymers (e.g. styrene), acrylates and/or methacrylates. The polymeric material may optionally be crosslinked, for example by
20 incorporation of cross-linking agents, for example as comonomers, e.g. divinylbenzene (DVB) or ethyleneglycol dimethacrylate. Appropriate quantities of the cross-linking agents (e.g. comonomers) required will be well known to the skilled man. Preferably the polymer is a
25 cross-linked styrenic polymer (e.g. a styrene-divinylbenzene polymer, surface functionalized by the use of a nitro-group containing comonomer, e.g. nitro-styrene, and subsequent reduction) or a cross-linked (meth)acrylic polymer surface functionalized by the use
30 of an epoxy-group containing comonomer (e.g. glycidylmethacrylate) and subsequent amination (e.g. by reaction with ethylene diamine).

The superparamagnetic crystals in the polymer particles used in the process of the invention may be of
35 any material capable of being deposited in superparamagnetic crystalline form in the porous polymer particles. Magnetic iron oxides, e.g. magnetite or

maghemite are preferred; however the crystals may be of mixed metal oxides or other magnetic material if desired. The total quantity of crystalline magnetic material present is generally more than 1%, preferably more than 3%, desirably more than or equal to 5% (by weight, e.g. up to 40% wt. The percentage is calculated on a Fe (or equivalent metal in the case of magnetic materials other than iron oxides) weight basis based upon the overall dry weight of the coated particles.

Polymer particles according to the various aspects of the present invention will generally have sizes (i.e. diameters) that are generally in the micrometer range, e.g. 0.3 to 100 μm , especially 0.5 to 50 μm , more especially 0.8 to 8 μm , e.g. 0.8 to 1.2 μm .

Typically the porous particles used will have a surface area of at least 15 m^2/g (measured by the BET nitrogen absorption method), and more preferably at least 30 m^2/g , e.g. up to 700 m^2/g , when corrected to a mean particle diameter of 2.7 μm (i.e. multiply surface area by 2.7/MD, where MD is the mean diameter in micrometers). Similarly scaled, the particle pore volume is preferably at least 0.1 mL/g .

Typically, the polymer particles are spherical and substantially monodisperse before they are coated and especially preferably remain spherical and substantially monodisperse once they have been coated.

By substantially monodisperse it is meant that for a plurality of particles (e.g. at least 100, more preferably at least 1000) the particles have a coefficient of variation (CV) of less than 20%, for example less than 15%, preferably less than 12%, more preferably less than 11%, still more preferably less than 10% and most preferably no more than about 8%, e.g. 2 to 5%. CV is determined in percentage as

$$\text{CV} = \frac{100 \times \text{standard deviation}}{\text{mean}}$$

where mean is the mean particle diameter and standard deviation is the standard deviation in particle size. CV is preferably calculated on the main mode, i.e. by fitting a monomodal distribution curve to the detected particle size distribution. Thus some particles below or above mode size may be discounted in the calculation which may for example be based on about 90% of total particle number (of detectable particles that is). Such a determination of CV is performable on a Coulter LS 130 particle size analyzer.

Functionalisation of the polymeric material may take place after polymerisation by, for example, nitration and subsequent reduction of the thus-formed nitro groups to pendant amine groups; or direct amination, for example by treatment with amino ethanol. As further alternatives, polymeric particles prepared by the well-known Ugelstad two-step swelling process and the improvements thereto disclosed in WO 00/61647 (Dyno) may be used. Porous polymer particles produced according to the processes described in this publication may have magnetic particles deposited in their pores by standard techniques.

As a further possibility, porous polymer particles may be prepared from nitro styrene and DVB, and magnetic material introduced as taught in US-A-4,654,267.

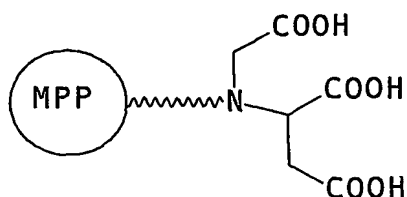
The superparamagnetic polymer beads sold by Dynal Biotech ASA under the trade names Dynabeads, especially Dynabeads MyOne are especially preferred. Dynabeads are particularly advantageous since they remain in suspension and do not exhibit magnetic particle sedimentation often associated with other magnetic beads. Dynabeads also show excellent magnetic mobility compared to other magnetic particles in which high levels of iron are present. Dynabeads exhibit beneficial kinetics allowing shorter reaction times and higher throughputs. Their unspecified binding is lower

than other magnetic beads and their proper use results in a concentration of the desired material taking place resulting in easier and more efficient washing procedures. Finally Dynabeads, e.g. MyOne beads
5 are easy to automate and are monodisperse.

The Cm-Asp ligand is bound to the magnetic polymer particle. By bound is meant that the ligand is covalently linked to the polymer particle, optionally using a linking group as discussed in detail below. The
10 Cm-Asp ligand can be bound to the magnetic polymer particle by various procedures although it is preferred if there are at least three linking atoms between the polymer particle surface and the nitrogen atom of the Cm-Asp. Preferably there are at least 5 atoms
15 separating the Cm-Asp ligand from the magnetic polymer particle surface, more preferably there are between 6 and 20 atoms separating the Cm-Asp ligand from the magnetic polymer particle surface.

The atoms forming the surface of the magnetic
20 polymer particle are those on the surface just prior to coupling with the linker/Cm-Asp ligand. Thus, if the magnetic polymer particle is activated in some way during its manufacture (e.g. nitrated and reduced to form an amino functionalised surface) the magnetic
25 polymer particle surface is formed by the surface nitrogen atoms. The nitrogen atom would form the first atom of the linker between the Cm-Asp ligand and the particle. In a magnetic particle functionalised to carry an electrophilic surface, e.g. a bromide surface,
30 the first atom of the linker would be that which displaces the bromine.

Hence, in a preferred embodiment the invention provides a conjugate of formula (I)



(where MPP is a magnetic polymer particle and the wavy line represents a linking group comprising at least
 5 three atoms, e.g. 3 to 20 atoms) or an analogue thereof in which a metal ion is chelated.

In US 6242581 aspartic acid is coupled to the solid phase prior to carboxymethylation to form the Cm-Asp ligand however it has not been possible to use this
 10 technique to provide a Cm-Asp group on a magnetic polymer particle. Rather, the inventors have devised alternative syntheses in which the Cm-Asp ligand is fully formed prior to coupling to the magnetic polymer particle.

15 In this regard, it has been found that when there are fewer than 3 atoms between the polymer surface and Cm-Asp ligand then coupling yields are low. In contrast to an agarose support carrying Cm-Asp (as describe in US-A-5962641), it is necessary in the present invention
 20 to ensure that coupling yields between the magnetic polymer particle and Cm-Asp are relatively high. The surface area of an agarose support is considerably greater than that of a polymer particle and hence the binding of Cm-Asp to the (agarose) support does not need
 25 to be achieved in high yield for a useful IMAC chelating agent to result. In the present case, yields need to be much higher to ensure that enough polymer particles carry the Cm-Asp ligand and hence to ensure that IMAC can be successfully carried out.

30 It is preferred if the at least 3 atom linker comprises an amino group (-NH-). Magnetic polymer beads are often made from styrene polymers which are nitrated to form NO₂ groups on the surface. After reduction of

these groups, e.g. using ammonia, amino groups are formed.

The linker thus preferably comprises the residue of an electrophile, i.e. the group which remains after
5 reaction of the electrophile with a nucleophile. Hence, the linker may comprise an oxo group ($C=O$, the residue of an ester/carboxyl group), a $-CH(OH)CH_2-$ group (the residue of an epoxide), $-CH_2-$ (where the electrophile is, for example a CH_2Hal). The linker may also incorporate a
10 number of atoms linking the actual electrophilic group to the nitrogen atom of the Cm-Asp ligand, e.g. an alkylene chain or ether chain, e.g. as in $-CH_2CH_2CH_2-$, or $-CH_2CH_2CH_2-O-$.

Hence the wavy line in formula (I) can represent -
15 $NH-Er-N-$ wherein Er represents a 2 to 20 atom linker which is an electrophile residue (Er), e.g. $-(CH_2)_n-$ where n is 2 to 20, which links the amino group of the particle surface with the nitrogen atom of the Cm-Asp ligand.

20 It is of course within the scope of the invention for the magnetic polymer particle to carry an electrophilic group with the Cm-Asp being functionalised to carry a nucleophilic group. Other ways of coupling the particle and ligand will be devised by the skilled
25 chemist.

In an especially preferred embodiment, a particle coating is provided which carries a carbon-carbon double bond. This can be achieved by, for example, reaction of the particle with an allyl or vinyl compound, e.g.
30 butenoic acid. Hydroxy functionalised particle surfaces can be reacted with allyl bromide to form double bonds on the particle surface. Also, carboxy functionalised particle surfaces can be reacted with allylamines to provide double bonds on the particle surface. The Cm-Asp
35 may then be coupled directly to the double bond using appropriate chemistry or more preferably, the double bond may then be reduced e.g. in the presence of aqueous

halide to provide a halide electrophile which can be reacted with the Cm-Asp ligand to ensure successful coupling.

Another preferred preparation process involves
5 functionalising the surface of the magnetic polymer particle to carry carboxyl groups. The carboxylic acid groups can be activated by reaction with N-hydroxysuccinimide esters and reacted with a Cm-Asp ligand as discussed above.

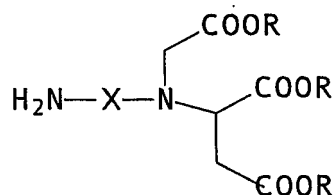
10 Suitable linkers therefore include aminoalkylene, amidoalkylene, ether, ester, thioalkylene or thioester containing up to 20 atoms, e.g. NH-alkylene, NH-CO-alkylene, O-alkylene, OCO-alkylene, S-alkylene or SCO-alkylene. The nitrogen atom of the Cm-Asp ligand does
15 not form part of the linker.

The Cm-Asp ligand may too be functionalised prior to coupling with the magnetic polymer particle. For example, it has proved advantageous to provide the Cm-Asp ligand with a linking group carrying a primary
20 nucleophile to aid reaction with electrophilic groups on the particle surface. The nitrogen atom of the Cm-Asp ligand is secondary and it has been found that this atom is too unreactive, perhaps due to steric hindrance, to react in high yield with electrophilic groups, e.g.
25 halides, on the particle surface.

It is preferred therefore to couple the Cm-Asp to a linker group having at least two atoms and comprising a nucleophile such as an amine, hydroxyl or thiol group. Preferably the linker is an alkylamine, e.g. C5/6-
30 alkylamine linker or an ether/polyether linkage e.g. comprising one or two oxygen atoms and 3 to 6 carbon atoms. Coupling of the linker to the Cm-Asp (via the nitrogen atom thereof) is achieved using known chemistry as described in the Examples. The Cm-Asp ligand itself
35 can be manufactured using known chemistry. It is also possible to synthesise the entire linker CmAsp structure.

Thus, the linker CmAsp structure can be prepared starting from a suitably protected aspartic acid compound, e.g. where the carboxyl groups are ester protected. This compound can be reacted with a compound
 5 of formula Hal-X₁-CN (where X represents a C₁₋₉ alkylene group, and Hal a halide, e.g. Br) wherein the amino group of the aspartic acid derivative displaces the halide atom. The resulting secondary amino compound may then be reacted with a Hal-CH₂COOPr type group (where Hal
 10 is halide, e.g. Br and Pr a protecting group) to introduce the final methylenecarboxy group to form the Cm-Asp structure. Selective reduction of the nitrile, e.g. using hydrogen and platinum (IV) oxide results in an ideal linker which can subsequently be deprotected as
 15 necessary.

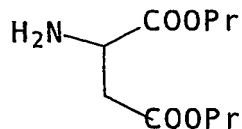
Thus, viewed from a further aspect the invention provides a process for the preparation of a compound of formula



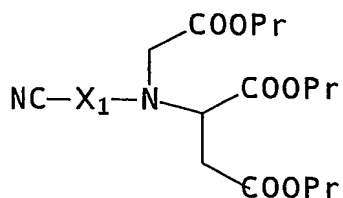
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(wherein each R independently represents hydrogen or a protecting group and X represents an C₂₋₂₀ alkylene linker, especially a C_{5/6}-alkylene);

comprising reacting a compound of formula Hal-X₁-CN
 25 (wherein Hal is a halide and X₁ represents an C₁₋₁₉ alkylene linker) with a compound of formula



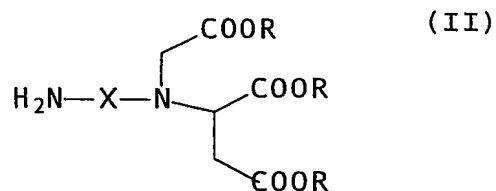
(wherein Pr represents a protecting group)
 30 reacting the resulting product with a compound of formula Hal-CH₂COOPr to form a compound



5 and reducing the nitrile to an amino group, preferably without removing the protecting groups. These can then be removed as necessary. The skilled chemist will realise that the X linker has one more carbon atom than the X₁ linker which derives from the nitrile.

10 The skilled chemist will be able to devise further methods for synthesising the Cm-Asp linker molecules of use in the invention.

Viewed from another aspect the invention provides a process for the preparation of a conjugate comprising a
15 magnetic polymer particle bound to a Cm-Asp ligand comprising reacting a Cm-Asp ligand of formula (II)

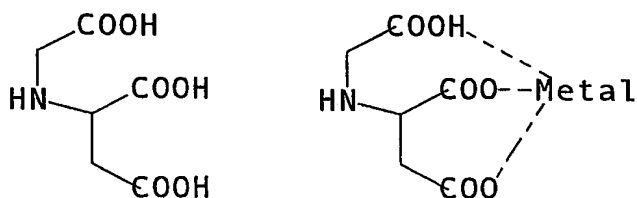


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(wherein each R independently represents hydrogen or a protecting group and X represents a 2 to 20 atom linker, e.g. an C₂₋₁₀ alkylene linker, especially a C_{5/6}-alkylene linker) with a magnetic polymer particle, e.g. one
25 functionalised to carry an electrophilic coating, e.g. an ester, epoxide, allyl, alkyl halide etc coating.

Compounds of formula (II) and analogues thereof in which a metal is chelated are themselves new and form a further aspect of the invention along with the Cm-Asp
30 ligand itself, i.e. a compound of formula (III) and its

analogue where a metal is chelated.



5 In some embodiments of the invention it may be necessary to protect the carboxyl groups of the Cm-Asp ligand during syntheses. This can be easily effected using known protection strategies, e.g. using an ester protecting group which can be hydrolysed in acid or base
10 as is known in the art.

The Cm-Asp ligand can coordinate any metal atom or ion. By metal is meant any metal from groups 1 to 13 of the periodic table, a lanthanide or actinide or a metal Si, Ge, Sn, Pb, As, Sb, Bi, Te, Po or At. The metal
15 should preferably be an ion and preferably be a transition metal or a metal of group 13. Preferred metal ions are those in the 2+ or 3+ oxidation states. Where the metal ion is in the 2+ oxidation state, the entire particle-linker-ligand-metal ion assembly is
20 uncharged which reduces the possibility of non-specific binding.

Preferred metals are Ni, Fe, Ga, Mn, Co, Cu and Zn of which Fe, Ga, Mn and Co are preferred, especially Co^{2+} . Coordination can be easily effected by exposing
25 the Cm-Asp to, for example, a metal chloride.

The conjugates with associated metal can in general be used for attaching to and combining with peptides, proteins or other polymers (e.g. antibodies) and are hence of use in a wide variety of assays. They are of
30 particular use, however, in the isolation of proteins/peptides tagged or native by immobilised metal ion affinity chromatography. In particular they are of use in the isolation of histidine-tagged recombinant

proteins/peptides, His, Cys, Met, Gln, Asn, Lys and/or Tyr containing native proteins and/or peptides, and phosphorylated proteins or peptides. Especially preferably, the conjugates are of use in the isolation of histidine-tagged recombinant proteins/peptides. Hence viewed from another aspect the invention provides the use of a conjugate comprising a magnetic polymer particle bound to a Cm-Asp ligand, said ligand coordinating a metal atom or ion, in an assay. Suitable assays and ways to carry these out are known by the skilled biochemist.

For example, the capture of histidine-tagged proteins on the Cm-Asp functionalised particles of the invention has various applications. The rapid reaction kinetics and gentle handling of isolated proteins make this technology well suited for the "pull down" of large protein complexes. Thus Cm-Asp functionalised beads may be used in sample preparation for mass spectrometry analysis. It is believed that complexes isolated with the Cm-Asp functionalised beads may be more intact than complexes isolated with columns or other solid supports including other magnetic particles with uneven surfaces and are therefore ideal for use in mass spectrometry sample isolation.

The Cm-Asp technology may also act as a solid phase for use in assay procedures. The Cm-Asp beads are not prone to aggregation and are highly dispersed in solution and show a low degree of non-specific binding. These properties allow for high quality screening results and protocols that are easily automated on a wide range of automation platforms. The Cm-Asp beads may also be used in phage display perhaps as a solid phase or to purify expressed phage display selected proteins from a library.

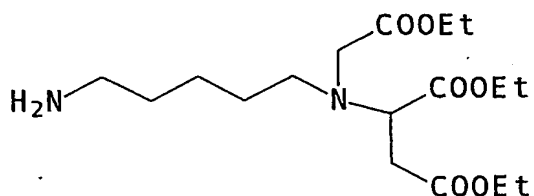
In general therefore the capture of histidine tagged proteins and/or His, Cys, Met, Gln, Asn, Lys and/or Tyr residue containing native proteins or

peptides may allow microscale protein purification,
clean up of mutated protein libraries, denaturing
elution of protein/peptide, mild elution of
proteins/peptide, protein-protein interaction studies
5 and screening technologies, e.g. for drug discovery,
molecular display, aptamer screening, phage display,
engineered enzyme screening and diagnostics.

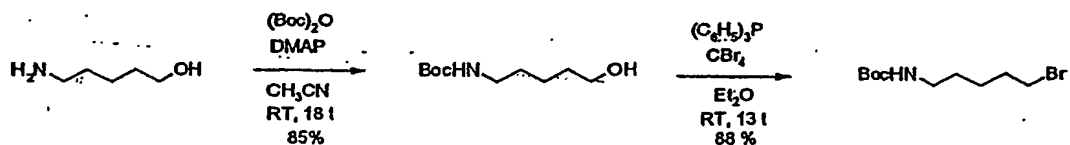
The invention will now be described further by
reference to the following non-limiting examples.

Example 1Synthesis of the Cm-Asp ligand

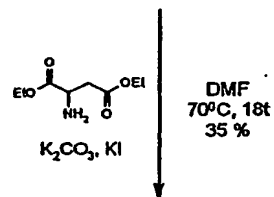
5 The Cm-Asp triester below is prepared as follows:



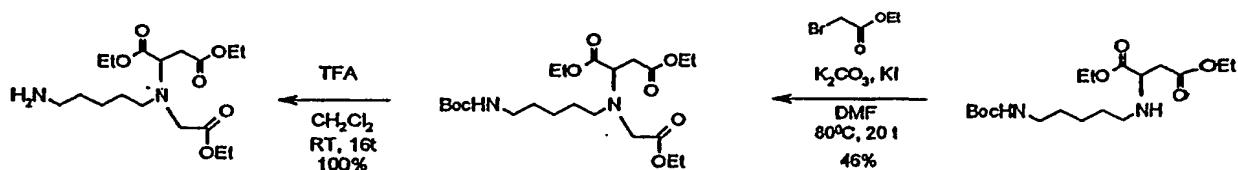
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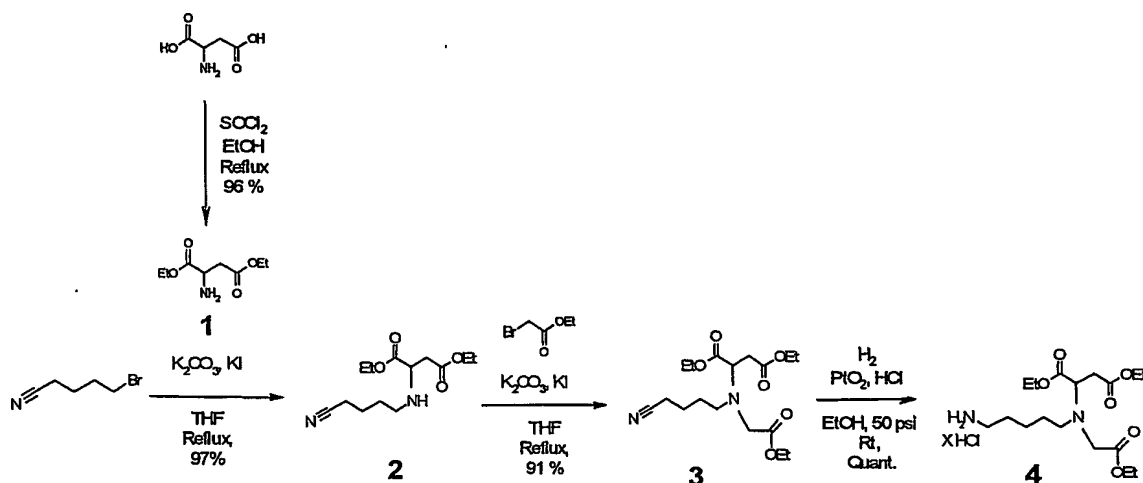
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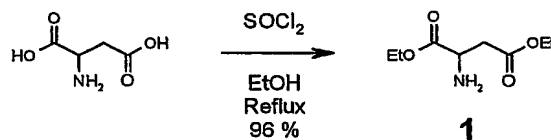
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Example 2Alternative Synthesis of Cm-Asp triester

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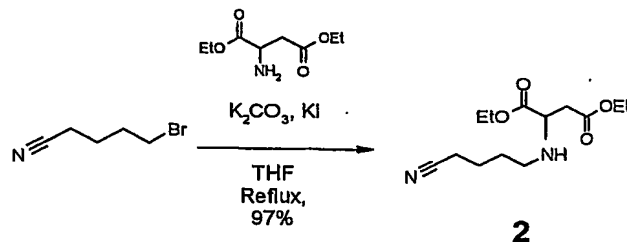
5 Synthesis of 2-amino-succinic acid diethyl ester



- 10 To a suspension of DL-aspartic acid (91.5 g, 0.69 mol) in abs. ethanol (800 ml) at 0°C thionylchloride (150 ml, 2.06 mol) was added dropwise. The cooling bath was removed and the mixture refluxed for 3 hours. After cooling to ambient temperature the solvent was
- 15 evaporated *in vacuo* and to the residue added a saturated aqueous solution of K_2CO_3 to pH 8. The aqueous phase was extracted with ethyl acetate (x 3) and the combined organic phases washed with brine and dried (MgSO_4), prior to filtration and evaporation *in vacuo* to give 124.8 g
- 20 (96 %) of compound 1 as an yellow oil. The crude product was used directly in the next step.

^1H NMR (200 MHz, CDCl_3): 4.06 (m, 4H), 3.68 (m, 1H), 2.61 (m, 2H), 1.73 (s, 2H), 1.06 (m, 6H).

Synthesis of 2-(4-cyano-butylamino)-succinic acid diethyl ester



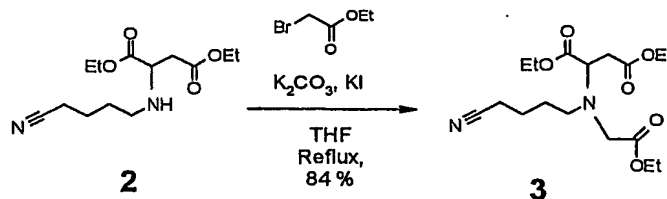
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To a suspension of **1** (93.0 g, 0.49 mol), K_2CO_3 (34.0 g, 0.25 mol), and KI (12.3 g, 0.07 mol) in THF (600 ml) 5-bromovaleronitrile (28.4 ml, 0.25 mol) was added dropwise. The reaction mixture was heated to reflux and stirred for 5 days. After cooling to ambient temperature the mixture was filtered, and the filtrate evaporated in vacuo. Purification on silica gel, eluting with hexane/ethyl acetate (7:3) afforded 64.1 g (97 %) of compound **2** as a yellow oil.

15

1H NMR (200 MHz, $CDCl_3$): 4.06 (m, 4H), 3.40 (t, 1H), 2.50 (m, 4H), 2.25 (t, 2H), 1.45 (m, 4H), 1.15 (m, 6H).

Synthesis of 2-[(4-cyano-butyl)-ethoxycarbonylmethyl-amino]-succinic acid diethyl ester



To a mixture of **2** (86.6 g, 0.32 mol), K_2CO_3 (44.3 g, 0.32 mol), and KI (16.0 g, 0.10 mol) in THF (650 ml) ethyl bromoacetate (42.5 ml, 0.38 mol) was added. The reaction mixture was heated to reflux and stirred for 5 days.

After cooling to ambient temperature the mixture was filtered, and the filtrate evaporated *in vacuo*.

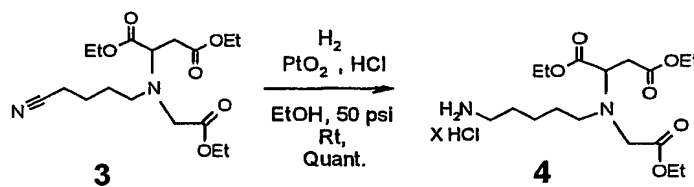
Purification on silica gel, eluting with hexane/ethyl acetate (8:2) afforded 103.7 g (91 %) of compound 3.

5

¹H NMR (200 MHz, CDCl₃): 4.18 (m, 6H), 3.91 (t, 1H), 3.42 (s, 2H), 2.77 (m, 4H), 2.40 (t, 2H), 1.65 (m, 4H), 1.25 (m, 9H).

10

Synthesis of 2-[(5-amino-pentyl)-ethoxycarbonylmethyl-amino]-succinic acid diethyl ester



15

To a solution of 3 (15 g, 42 mmol) in 95% ethanol (60 ml) and concentrated HCl (10 ml) a suspension of PtO₂ (600 mg, 2.6 mmol) in 95% ethanol (20 ml) was added. The reaction mixture was hydrogenated at 50 psi overnight. The mixture was filtrated and the filtrate evaporated *in vacuo* and pumped overnight to afford a quantitative yield of the title compound as the HCl-salt.

20

¹H NMR (200 MHz, D₂O): 4.82 (t, 1H), 4.20 (m, 6H), 3.53 (q, 4H), 3.34 (m, 2H), 3.18 (b d, 2H), 2.92 (b t, 2H), 1.65 (m, 4H), 1.10 (b m, 9H).

25

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Example 3:
Bromination

17.3 g of a methanol suspension of the magnetic styrene particles having 0.5 mmol/g allyl groups were washed four times with 45 mL sodium acetate buffer (pH = 5.9). After adjusting the particle content to 9 wt%, 0.96 g of
5 pyridinium tribromide dissolved in 10 mL DMF was added while stirring at 350 rpm. After five minutes at room temperature the particles were washed five times with 45 mL deionised water.

10 **Example 4:**

Functionalization with Cm-Asp chelator

18.0 g of a suspension of the particles prepared as in Example 3 were washed three times with 20 mL of 50mM
15 sodium bicarbonate. The particle content was adjusted to 12 wt%. To the suspension 0.17 g of the Cm-Asp triester (prepared as described in Example 1) was added. 50mM sodium bicarbonate was added until a particle content of 10 wt% was achieved. The reaction mixture was shaken at
20 600 rpm at 40°C for 15 hours. The particles were then washed four times with 20 mL deionised water.

Example 5:

Hydrolysis

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20.0 g of a suspension of particles prepared as in Example 4 were washed twice with 20 mL of 1M lithium hydroxide. After adjusting the particle content to 10 wt% the mixture was shaken at 250 rpm for four hours at
30 room temperature. The particles were then washed with deionised water until pH 6-7.

Example 6:

General Metal-loading conditions

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250 mg of particles prepared as in Example 5 are washed

twice with 5 ml reverse osmosis-water followed by 15 min sonication. 5 ml of 10 mM metal salt (MX) are added to the particles and incubated for 30 min. The tube is placed in a magnet, and the supernatant is removed. The particles are washed twice with 5 ml phosphate buffered saline (0,01% Tween 20, pH 7,4). The particles are then washed once in 20% ethanol. The particles are stored in 20% ethanol.

10 The following metal salts were employed MX = CoCl_2 , CuSO_4 , FeCl_3 , GaCl_2 , GaCl_3 , MnSO_4 , MgCl_2 , NiCl_2 , CaSO_4 , ZnCl_2

Example 7

15 Functionalisation of carboxylic acid groups to N-hydroxysuccinimide ester

50 g of a suspension of 5.0 g of the particles of MyOne Carboxylic acid beads (Dynal Biotech ASA) were acidified by washing with 0.1 M acetic acid (3 x 50 mL). The acidified particles (which have a carboxylic acid content of 0.5 mmole/g DS) were then washed with acetone (4 x 50 mL) and concentrated on a magnet. Extra acetone was added until a total of 35.6 g suspension is achieved. N-hydroxysuccinimide (2.90 g, 25 mmole) and diisopropylcarbodiimide (3.16 g, 25 mmole) were then added. The reaction mixture was stirred at room temperature for 5 hours. The particles were then washed with acetone (5 x 50 mL).

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Example 8:

Functionalization with Cm-Asp chelator

44 g of an acetone suspension of the beads of Example 7, were washed three times with 50 mL isopropanol. After adjusting the particle content to 12 wt%, 5.6 g of

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triethylamine was added. 0.10 g of the Cm-Asp triester (prepared as described in Example 1) dissolved in isopropanol, was then added. This results in a particle content of 10 wt%. The reaction mixture was then shaken
5 at 250 rpm at room temperature for 20 hours. The particles were washed three times with 50 mL of isopropanol.

Example 9:

10 Functionalization with Cm-Asp chelator and ethanolamine

To 10 g of an isopropanol suspension of the particles prepared as in Example 8, 0.32 g of ethanolamine was added. The reaction mixture was then shaken at 250 rpm
15 at room temperature for 18 hours. The particles were then washed three times with 10 mL of isopropanol.

Example 10

Functionalization with Cm-Asp chelator

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1,2 gram of dry Dynabeads 270 Epoxy were mixed with 8,8 gram of 50 mM sodium bicarbonate. To the suspension 0,17 grams of the Cm-Asp triester (prepared as described in Example 1) were added, and the reaction mixture was
25 shaken at 600 rpm at 60°C for 16 hours. The particles are worked up by washing four times with 20 ml deionised water.

Example 11

30 Purification of Histidine-tagged recombinant proteins

1. 2 mg of a suspension of particles with Co²⁺ prepared as in Example 6 were washed with 700 µl 50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 0.01% Tween®-20.
- 35 2. The supernatant was removed and the particles were resuspended in 100 µl of the same buffer as in step 1.

3. A suspension of lysed E. coli cells with expressed recombinant histidine-tagged protein was added to the particle suspension. The total volume was adjusted to 700 μ l with the same buffer as in step 1. This
5 suspension was incubated for 10 minutes.
4. The supernatant was removed and the particles with the bound histidine-tagged protein were washed four times with 700 μ l of the same buffer as in step 1.
5. The histidine tagged protein was eluted in 100 μ l
10 150 mM Imidazole, 50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 0,01% Tween®-20.
6. The purified protein was analysed by SDS-Tris-HCl polyacrylamide gel and bromphenol blue staining.

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Example 12Purification of phosphorylated peptides

1. 2 mg of a suspension of particles with Fe³⁺ prepared
20 as in Example 6 were washed twice with 500 ml 5% acetic acid.
2. The supernatant was removed and 100 μ l 10% acetic acid was added. 100 μ l of 30 μ g trypsinated b-casein was added. This was incubated for 30 min.
- 25 3. The supernatant was removed and the particles with the bound phosphorylated peptides were washed twice with 250 μ l 1% acetic acid.
4. The supernatant was removed and the particles with the bound phosphorylated peptides were washed twice with
30 250 μ l 0,1% acetic acid, 10% acetonitrile.
5. The supernatant was removed and the particles with the bound phosphorylated peptides were washed with 250 μ l H₂O.
6. The phosphorylated peptides were eluted in 50 μ l
35 0,1 M ammonium bicarbonate.
7. The purified phosphorylated peptides were analysed by HPLC.

Example 13Purification of metal binding proteins

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1. 2 mg of a suspension of particles with Mn^{2+} prepared as in Example 6 were washed twice with 250 μ l Acetate buffer pH 4.0, 250 mM NaCl.

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2. The supernatant was removed and 250 ml of the same buffer as in step 1 was added. 30 μ g (3 μ l) of an Mn binding protein was added. This was incubated for 10 min.

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3. The supernatant was removed and the particles with the bound protein were washed twice with 250 μ l of the same buffer as in step 1.

4. The Mn-binding protein was eluted in 50 μ l 0,1 M ammonium bicarbonate.

5. The eluted protein was analysed by SDS-Tris-HCl polyacrylamide gel and silver staining.

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